

Altered O^6 -Alkylguanine-DNA Alkyltransferase Activity in Cell Strains Originating from Mouse Skin Tumors Induced by UV Irradiation¹

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ABSTRACT

O^6 -Alkylguanine-DNA alkyltransferase (AGT) activity was assayed in the extracts of 47 cell strains originating from mouse skin tumors induced by UV irradiation. They were also examined for the sensitivity to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride by colony formation. The AGT activity (fmol/mg protein) of the tumor cell strains varied widely and the mean \pm SE was 72.5 ± 9.37 , while the AGT activity of the nontumor cell strains was 134 ± 17 . Among 47 strains, 6 strains showed extremely low or no AGT activity, about 5 fmol/mg protein or less, and were hypersensitive to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride. Long-term culture of the tumor cells did not change the AGT activity except in some strains which might have had coexisting normal cells in the population in early passages. All strains showed similar UV sensitivity regardless of AGT activity. This is the first report which demonstrates that about 13% of newly induced tumor cell strains are deficient in AGT activity similar to Mer⁻/Mex⁻ phenotype that was found in approximately 20% of the established human tumor cell strains.

INTRODUCTION

Alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-methyl-*N'*-nitrosourea, and ACNU³ produce O^6 -alkylguanine at O^6 of guanine in DNA (for review see Ref. 1). O^6 -Alkylguanine is known to be repaired by AGT (2, 3) which removes the alkyl group from O^6 -alkylguanine in cellular DNA. Involvement of O^6 -alkylguanine in mutagenesis (4, 5) and carcinogenesis (6-9) has been suggested, presumably through its mispairing with thymine during semiconservative DNA replication, yielding G:C-A:T transition (2, 10).

Approximately 20% (19 of 93) of human tumor cell strains (11) and 7 of 11 human fibroblast cell strains transformed by simian virus 40 (4) or about one-third (7 of 23) of human lymphoblastoid cell strains immortalized by Epstein-Barr virus (12, 13) were reported to be deficient in the ability to repair O^6 -alkylguanine in DNA. Such phenotype was termed Mer⁻ or Mex⁻ (4, 9, 11, 12), and the Mer⁻/Mex⁻ cells had no or extremely low activity of AGT (3, 4, 11, 14, 15). Some AGT-deficient cell strains were found to originate from tumors arising in patients whose normal fibroblasts showed the Mer⁺ phenotype (4).

The low AGT activity in the human tumor and virus-transformed cell strains could have arisen through the following processes: (a) cells with low AGT activity, preexisting as a minor population, may be more susceptible to neoplastic transformation than normal cells because they cannot repair the

DNA damage induced by the alkylating agents in the environment; (b) prolonged culture *in vitro* may influence the phenotypic states of repair as suggested by Scudiero *et al.* (16) and Arita *et al.* (13); (c) the Mer⁻/Mex⁻ phenotype appears to coincide with neoplastic transformation (cause or result) by unknown mechanisms. Mutation and selection may be involved in the latter two possibilities, but no supporting evidence has been presented so far. The direct involvement of alkylating agents in the production of Mer⁻/Mex⁻ phenotype in tumor cells is unlikely since the phenotype appeared often in virus-transformed cells.

The purpose of this study was to produce tumors with agents other than alkylating agents or viruses and to assess the Mer⁻/Mex⁻ phenotype of cell strains from them. We irradiated mouse skin with UV and skin tumors were produced. UV was chosen because the repair mechanisms of UV damage had been extensively studied and AGT is not involved in the repair process of UV damage (16). Cells were grown from the tumors, and AGT activity and ACNU sensitivity of the cells were measured to identify the Mer⁻/Mex⁻ phenotype. The frequency of appearance of cell strains with decreased AGT activity was estimated at early and late passages of cell culture originating from the tumors.

MATERIALS AND METHODS

Mice. Specific-pathogen-free hairless albino mice of the inbred strain HOS (HR/De) were supplied by Hoshino Experimental Animal Farm (Saitama, Japan). Strain HOS is a derivative of HRA/SKH-1 originally obtained from the Skin and Cancer Hospital, Temple University, Philadelphia, PA. BALB/c hairy albino mice were supplied by Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). They were given chlorinated water *ad libitum* and autoclaved mouse chow. Room illumination was on an automated cycle of 12-h light, 12-h dark. Room temperature was 22-25°C.

Chemicals. *N*-[methyl-³H]Nitrosourea (specific activity 1.0 Ci/mmol) was purchased from New England Nuclear (Spectra, Boston, MA). ACNU was purchased from Sankyo Co., Tokyo, Japan.

Production of Skin Tumor by UV Irradiation. The light source was a bank of 6 Toshiba FL20SE sunlamps with dose rate of 6.3 J/m²/s encompassing the wavelengths of UVB (280 to 320 nm) with a peak at 305 nm. Flux intensity was measured with UVR-305/365D digital radiometer (Tokyo Kogaku Kikai KK, Tokyo, Japan).

UV irradiation of mice started when mice were 7-8 weeks old. In hairless mice, 3430 J/m² of irradiation, 2-3 times a week for 19-22 weeks was the standard procedure. There are slight differences in irradiation procedures among experiments, and the details, including the process of tumor production and pathological identification of the tumors, will be described elsewhere.⁴ Tumors were designated HL1 through HL81. In hairy mice, the back was shaved once a week and irradiated with the sunlamps, 7620 J/m² per irradiation, 2-3 times a week. Tumors in the hairy mice were designated HR1 through HR12.

Tumor Transplantation to Nude Mice. Tumors were removed, cut into small pieces (about 1-2 mm in diameter), and 5-6 pieces were transplanted to a nude mouse *s.c.* in each side of the flank with a trocar.

Culture of Tumors. Small pieces of tissue (5 x 5 mm) were excised from tumors without visible necrosis or infection. These pieces were

Received 4/20/90; accepted 2/22/91.

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¹ This work was supported by Grants-in-Aids from Ministry of Education, Science and Culture, Japan.

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³ The abbreviations used are: ACNU, 1-(4-amino-2-methyl-5-pyridinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; AGT, O^6 -alkylguanine-DNA alkyltransferase; D₃₇, dosage (μ g/ml) required to achieve 37% survival.

⁴ C. Nishigori *et al.*, manuscript in preparation.

immersed in culture medium containing antibiotics for 30–60 min, 2–3 times. They were minced into 0.5- to 1-mm³ pieces with a sharp scalpel, and 5–6 pieces were placed in plastic 25-cm² flasks with a small amount of culture medium, which consisted of Dulbecco's modified Eagle's minimum essential medium (Nissui; Tokyo, Japan) supplemented with 15% fetal calf serum (HyClone Laboratory, Inc., Logan, UT). When cells reached confluency, usually after 2–3 weeks, they were transferred to 75-cm² flasks and used for experiments. Normal mouse skin fibroblasts were grown by a similar procedure from 6- to 7-week-old mice and designated SKHI through SKH5. We obtained 47 tumor cell strains out of 93 attempts and 5 normal cell strains. Detailed characteristics of the tumors will be published elsewhere.⁴

ACNU Survival of Cultured Tumor Cells. Appropriate numbers of cells were inoculated into 6-cm dishes and incubated for 18 h. Cells were washed with medium and treated with serum-free medium containing ACNU for 1 h. Cells were further incubated in medium containing 15% fetal calf serum with a medium change twice a week until colony formation. After fixing and staining, colonies (50 cells or more) were scored.

UV Survival of Cultured Tumor Cells. Cells were grown and treated as above except for UV irradiation in place of ACNU treatment. Cells were rinsed with phosphate-buffered saline, aspirated, and irradiated with germicidal UV lamps (emission peak, 254 nm).

AGT Activity. Confluent cells in three 10-cm dishes were harvested by rubber policeman and suspended in 2 ml of 70 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.1) containing 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. The cell suspension was sonicated for 20 s in ice and centrifuged. The supernatant was stored at –80°C until use. The protein content of the cell extracts (supernatant) was measured by the Bio-Rad assay. The cell extract was mixed with the reaction substrate (salmon sperm DNA treated with [methyl-³H]-nitrosourea) prepared as described previously (17) and incubated for 1 h at 37°C. Ice-cold trichloroacetic acid (5% final concentration) was added to the reaction mixtures, which were kept on ice for 15 min and then heated at 80°C for 30 min. The precipitated protein was collected on a GF/C filter (Whatman Ltd., Maidstone, United Kingdom) and washed with trichloroacetic acid and ethanol. Radioactivity remaining on the filter was measured with a liquid scintillation counter.

RESULTS

AGT Activity of Skin Tumor Cell Strains. AGT activity of the tumor cell strains originating from the UVB-irradiated hairless (HL) mice in early passages varied widely as shown in Fig. 1. AGT activity in other tumor cells of the hairless mice not shown in Fig. 1 and those originating from hairy mice had similar variation in AGT activity as shown in Fig. 1. The enzyme activity increased in proportion to the amount of protein. For quantitative comparison, relative AGT activities in cell extracts were calculated from the linear portions of the curves. The activity is expressed as fmol of methyl residues which were removed from DNA per mg protein during 1 h. Distribution of AGT activity in the tumor cells is shown in Table 1. The mean value of AGT activity (fmol/mg protein) in the tumor cells in early passages (less than passage 10) was 72.5 ± 9.37 (n = 47) while that of nontumor cells was 134 ± 17 (significantly higher by Student's *t* test; *P* < 0.05). Most of the tumor cells showed lower AGT activity than nontumor cells with the lowest AGT activity. Among them, 5 tumor cell strains were very reduced in AGT activity, about 5 fmol/mg protein or less. Table 1 also indicates the transplantability of the tumors in nude mice; the tumors with high AGT activity appeared to be less susceptible to transplantation and longer time is needed for detectable tumors to develop after transplantation. AGT activity of the cells did not appear to depend on the growth rate *in vitro* or on the histological nature of the tumors.

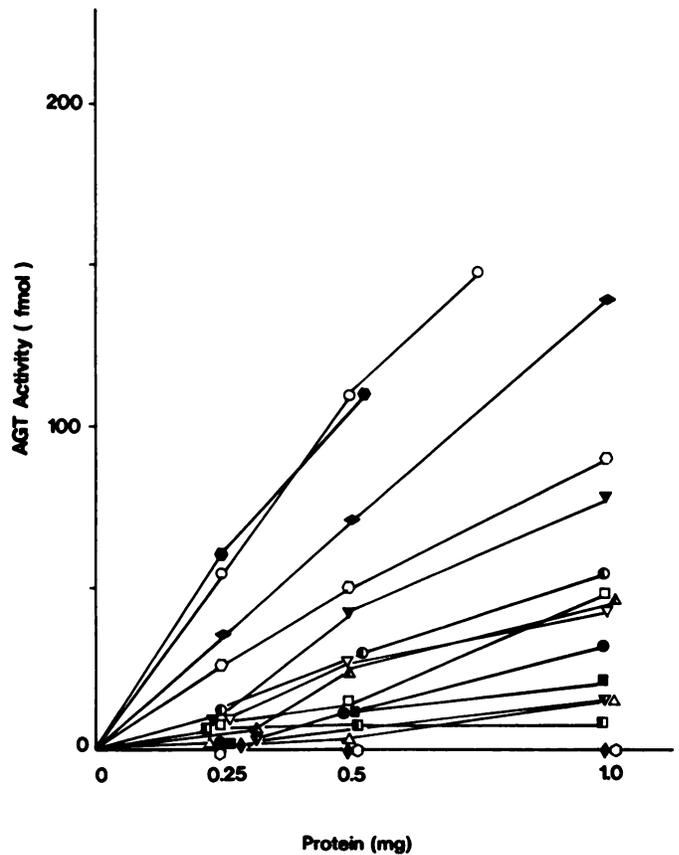


Fig. 1. AGT activity (fmol/mg protein with 1 h incubation) in early passage cell cultures originating from UVB-induced skin tumors in SKH hairless mouse. Cell passages (P) are given in parentheses. HL1 (P-6) (□); HL10 (P-4) (◆); HL21 (P-7) (■); HL25 (P-6) (Δ); HL29 (P-4) (▨); HL32 (P-7) (▽); HL34 (P-5) (○); HL43 (P-61) (●); HL50 (P-4) (△); HL60 (P-4) (◆); HL61 (P-5) (▽); HL62 (P-7) (▼); HL63 (P-6) (○); HL67 (P-6) (●); HL70 (P-5) (○); SKH 1 (P-4) (○).

Table 1 AGT activity in tumor cells and transplantability of tumors

AGT activity in early passage ^a (fmol/mg protein)	No. of tumors	Transplantable tumor ^b
<10	5	3/3
10–50	17	14/15
50–100	12	12/12
>100	13	10/13
Total	47	39/43

^a AGT activity was calculated from the linear portion of the curves as shown in Fig. 1. The reaction mixture was incubated for 1 h.

^b Some of the tumors were not tested for transplantability due to small size.

Effect of Long-Term Culture *in Vitro* on AGT Activity. AGT activity of 10 cell strains at early and late passage was compared. No remarkable change was noted except in one strain, HL8 (Table 2), which had intermediate AGT activity in the early passages and very reduced AGT activity in the late passages. HL8 cells were very sensitive to ACNU in colony-forming ability at passages 34 and 71 (see Fig. 2), and their ACNU sensitivity in passage 4 was not measured due to poor plating efficiency. HL8 cells at passage 4 appeared to be heterogeneous morphologically. In another cell strain, HL6, both AGT activity and ACNU sensitivity were measured at passages 5 and 106. HL6 cells in passage 106 were reduced in AGT activity to 33% of that in passage 5, and ACNU sensitivity was 2-fold more sensitive in passage 106 than in passage 5 when the slopes of the curves were compared. Five other cell strains also exhibited lower AGT activity in the late passages than in the early

Table 2 Changes in AGT activity during passages

Cell strain	AGT of early passage (a) ^a		AGT of late passage (b)		b/a
	Passage no.	AGT activity	Passage no.	AGT activity	
HL8	4	62 (1)	71	1.8 ± 1.1 (6)	0.03
HL34	5	2.2 ± 0.4 (3)	43	4 (1)	1.81
HL5	4	18 ± 2 (2)	19	11 ± 2 (3)	0.60
HL40	5	39 ± 2 (2)	36	16 ± 1 (3)	0.41
HL9	4	43 ± 0.9 (2)	72	21 ± 2 (3)	0.49
HL7	3	54 ± 2 (2)	118	21 ± 1 (3)	0.39
HL6	4	113 ± 2 (3)	106	37 ± 4 (3)	0.33
HL3	5	35 ± 3 (2)	65	46 (1)	1.32
HL2	8	85 ± 10 (2)	65	54 ± 10 (4)	0.63
HL13	5	113 (1)	30	148 ± 4 (2)	1.31

^a Early passage (a) and late passage (b), AGT activity (fmol/mg protein) is indicated by mean ± SE of several experiments. Numbers in parentheses, number of experiments.

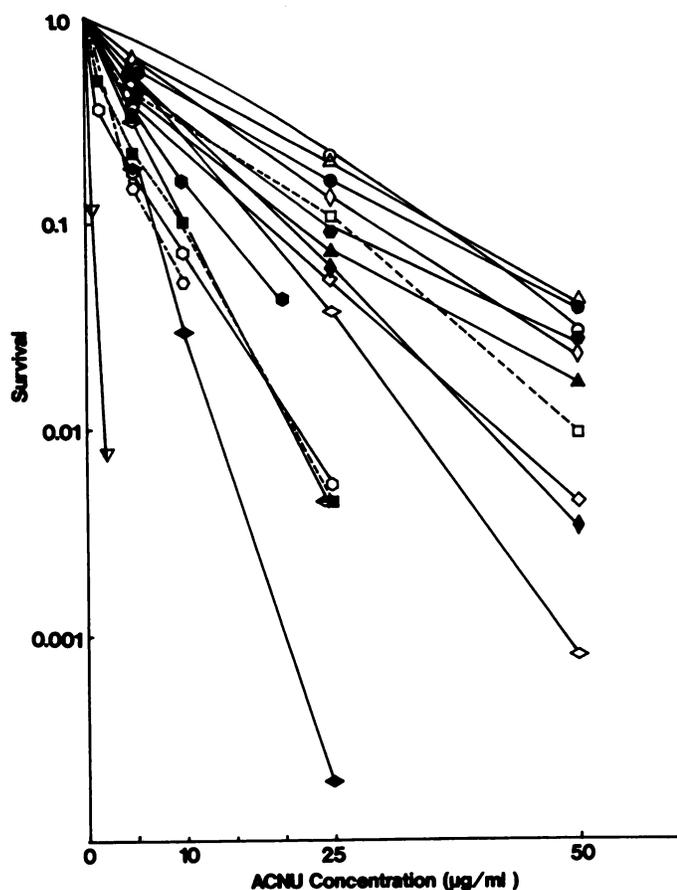


Fig. 2. ACNU survival curves of the tumor cell strains. Numbers of passages (P) are indicated in parentheses. HL5 (P-31) (◆); HL7 (P-118) (); HL8 (P-71) (); HL9 (P-72) (); HL10 (P-5) (Δ); HL11 (P-5) (◇); HL13 (P-30) (◇); HL21 (P-7) (●); HL34 (P-43) (); HL58 (P-7) (○); HL60 (P-6) (○); HL62 (P-3) (●); HL78 (P-2) (○); HR7 (P-5) (■); SKH 1 (P-4) (▲); SKH 2 (P-3) (●); BALB/c (P-2) (□). The survival curve for HL8 cells in passage 34 overlapped exactly with that in passage 71.

passages, while slight elevation in the AGT activity in later passages was noted in 3 cell strains (Table 2). Cells at late passages (passages 30–118) appeared to be beyond the crisis period because they were round in shape and easily detachable from the dishes, and growth was very rapid.

ACNU Sensitivity. Sensitivity to ACNU was assayed by colony formation, and survival curves for 16 tumor cell strains and 3 nontumor cell strains are shown in Fig. 2. Twelve other tumor cell strains not shown in Fig. 2 were within the range of the

sensitivity of the majority of cell strains in Fig. 2, excluding HL8. D₃₇ values (µg/ml for 37% survival) varied widely among the tumor cells (0.5 < D₃₇ < 14.3), while those of the nontumor cells were between 11 and 15. Plating efficiency of the tumor cell strains assayed for ACNU sensitivity were 5 to 32% and other tumor cell strains with plating efficiency less than 5% were not tested for ACNU sensitivity (19 strains). One strain, HL8, with almost no AGT activity in late passage (Table 2) was extremely sensitive to ACNU (Fig. 2). Four other cell strains (HL34, HL58, HL60, and HR7), about 5 fmol/mg protein or less in AGT activity, were relatively more sensitive than most of the other cell strains.

UV Sensitivity. The UV sensitivities of 20 tumor cell strains and 2 nontumor cell strains were compared and the variation in sensitivity was far less than that in ACNU sensitivity. Cell strains with high ACNU sensitivity were not different from other strains in UV sensitivity.

Correlation between ACNU Sensitivity and AGT Activity. Twenty-six tumor cell strains and two nontumor cell strains were assayed for both ACNU sensitivity and AGT activity. ACNU sensitivity is expressed in D₃₇, and AGT activity is in fmol/mg protein. The relationship between these two characteristics is shown in Fig. 3. AGT activity correlated well with cellular resistance to ACNU (assuming a linear correlation, Y = 0.536X, where Y is ACNU D₃₇ and X is AGT activity in fmol/mg protein; correlation coefficient is 0.808, n = 28; significantly correlative, P < 0.005).

Some of tumor cell strains, for example, the HL3:HL5 and HL10:HL11 pairs, shown in Fig. 3, originated from the same mouse. In a pair of HL41 and HL43, from a mouse, not shown in Fig. 3 due to poor plating efficiency, AGT activities differed 11-fold.

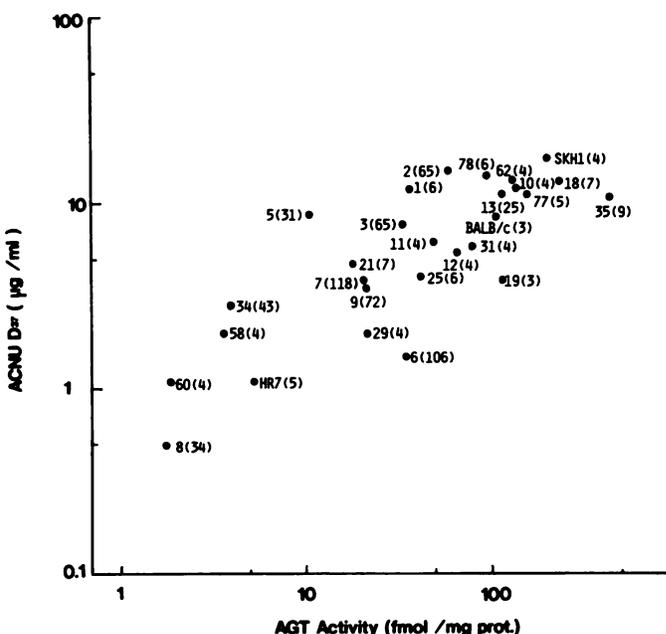


Fig. 3. Relationship between AGT activity (fmol/mg protein) and ACNU concentrations (µg/ml) for D₃₇ on ACNU survival in cultured tumor cells in the same passages. Numbers of passages are indicated in parentheses. Names of the tumor cell strains are given in numbers for hairless mice and numbers with HR for hairy mice.

DISCUSSION

Wide variation was demonstrated in the AGT activity among cell strains established from UV-induced mouse skin tumors, with good correlation with ACNU sensitivity. This is in agreement with the variation in the AGT activity in human tumor cell strains (18, 19). Six tumor cell strains, 5 in early passages (Table 1) and HL8 in late passages (Table 2), had extremely low (about 5 fmol/mg protein or less) AGT activity similar to that in human Mer⁻/Mex⁻ cells. Although no clear distinction between the mouse tumor cell strains corresponding to Mer⁺/Mex⁺ and the Mer⁻/Mex⁻ phenotypes can be drawn, the next higher AGT activity in the cell strains in comparison with the above 6 strains was 11 fmol/mg protein (Table 2; Fig. 3). AGT activities in these 6 cell strains were as low as those classified to be the AGT-deficient mouse cells by Yagi *et al.* (20). Our data represent the first successful conversion from normal to AGT-deficient phenotype similar to the Mer⁻/Mex⁻ by an agent other than viruses. The frequency of the strains with such phenotype, 6 of 47 or 13%, is comparable to those in previous reports on human tumor cell strains (11, 18). Virus-transformed cells were reported to be more susceptible to conversion to Mer⁻/Mex⁻ phenotype (3, 10). Such a high frequency of conversion to AGT-deficient, or the Mer⁻/Mex⁻, phenotype in the absence of known selection factors implies that transformation or tumorigenesis may have caused the change in AGT activity. Normal cultured human diploid or primarily cultured mouse cells have never been shown to be deficient in AGT activity (4, 14, 20), indicating that conversion to Mer⁻/Mex⁻ may well be associated with transformation or tumorigenesis. Our work strongly supports this interpretation and the frequency of conversion agrees with that found in the established tumor cell strains. What factor is involved in the conversion remains to be discovered. Our preliminary analysis suggests that when cells were grown from large skin tumors, they were often reduced in AGT activity, and they grew fast when they were transplanted in athymic nude mice. Although this tendency is not quantitatively confirmed, conversion to AGT-deficient phenotype could be related to the degree of malignancy of the tumors.

The conversion to AGT-deficient cell strains with UV irradiation, as well as by virus, excludes the involvement of alkylating agents in causing tumorigenesis through neoplastic transformation of preexisting AGT-deficient cells as a minor population. One possible mechanism of conversion to Mer⁻/Mex⁻ cells is that the changes take place during cell culture, not in tumor *in vivo*, since frequency of Mer⁻/Mex⁻ phenotype in tumor tissue appeared to be lower than that in cultured tumor cells (21, 22).

Comparison of AGT activities between cells in early and late passages might indicate that the AGT activity becomes low during repeated passages. One strain, HL8, became essentially defective in AGT activity in passages 34 and 71, while it had nearly normal level of AGT activity in passage 4. In passage 4, cells appeared to be very heterogeneous morphologically and the plating efficiency was extremely low. In passages 34 and 71, HL8 cells were more homogeneous morphologically and smaller than those in passage 4, and grew very rapidly. It may be reasonable to assume that a small fraction of AGT-deficient cells in passage 4 outgrew other cells during cell culture and passages. Cell strain HL34 was already AGT deficient in passage 5, and less heterogeneous morphologically than strain HL8 in passage 4. This cell strain retained the AGT deficiency in passage 43. Other 8 tumor cell strains showed slight changes

in AGT activity, 6 reduced and 2 enhanced, by increasing passages (Table 2). Measuring the AGT activity in the tumors before starting them in culture was not tried, mainly because of difficulty in isolating the tumor cells only, since the tumor and surrounding tissue were intermingled. These results may exclude the possibility that the conversion to AGT-deficient phenotype is induced during cell culture and passage. AGT-deficient cells may have existed in early passages, originating from the tumors, and might become predominant in late passages by slight growth advantage.

Some of the tumor cell strains that originated from the same mouse showed widely variant AGT activity. For example, strain HL41 is 11-fold higher in AGT activity than strain HL43, originating from the same mouse. This indicates that alteration in AGT activity does not depend on the genetic background and may represent the characteristics of the tumor.

In conclusion, tumors produced by UV irradiation yielded about 13% cell strains with very low AGT activity, similar to the Mer⁻/Mex⁻ phenotype. Conversion to this phenotype should be associated with tumorigenesis through unknown mechanisms which may not be influenced by cell culture or passage *in vitro*.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Kouichi Tatsumi for valuable discussion, Dr. Tohru Tsujimura for technical advice on the AGT assay, Dr. Junko Tatsumi for advice for statistical analysis, and Dr. Miran Tanaka for help in animal experiments.

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